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Exploring the microbiome: diversity of the microbial community of three foam nesting frogs, Genus: Polypedates, across a developmental gradient

Sarah McGrath

Exploring the microbiome: diversity of the microbial community of three foam nesting frogs, Genus: *Polypedates*, across a developmental gradient

Sarah McGrath

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FACULTY COMMITTEE:
Committee Chair: Dr. David S. McLeod
Committee Members/Readers:
Dr. Morgan Steffen
Dr. Reid Harris

Dedication

This work is dedicated to my father, John Robinson McGrath, for instilling in me a love of nature; my mother, Susanna Terrell McGrath, for continuously demonstrating that hard work pays off; Nicholas Blaser, for being an unwavering source of support; and Jennifer A. Sheridan, for providing me with my first opportunity to fully understand the demand and intricacy of tropical fieldwork.

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ABSTRACT

Characterization of microbial biodiversity, including that of the amphibian skin-associated microbiome, is a frontier of research recently made accessible through advances in sequencing technology. Microbial interaction with a host has been determined to have profound influences on host health across a wide range of macroscopic organisms. For amphibians, the influence of the skin-associated microbiome has been found to have particular importance, as amphibians are currently one of the fastest disappearing vertebrate groups on the planet, largely in part to skin-associated diseases caused by pathogenic microbes. Therefore, it is important to characterize the amphibian skin-associated microbiome, particularly for species with no existing microbiome data, and to delineate relationships that may influence host health. In determining the microbial community of amphibian skin, it is important to outline baseline native microbial presence and gain insight into how these microbes become established. This study focused on being the first to characterize the cutaneous microbial diversity of three Southeast Asian tree frogs in the family Rhacophoridae (genus: *Polypedates*) that reproduce via the specialized breeding strategy of building a foam nest and comparing the amphibian microbiome across initial development to that of the environment. Microbes associated with reproducing adults, foam nests, tadpoles before and after environmental interaction, and the surrounding environment were characterized using 16S amplicon sequencing. The phylum Proteobacteria comprised the majority of communities across amphibian and environmental samples at 57% relative abundance with Firmicutes (16%) and Bacteroidetes (13%) as the next most dominant phyla. In comparing amphibian and environmental samples, no amphibian microbial communities

mirrored that of their immediate environment. Interestingly, tadpole skin-associated microbes differed in relative abundance and microbial taxa between nest-extracted tadpoles and those that were sampled after interaction with a pond environment. This demonstrates the necessity of further research into microbial community establishment, host selection processes, and microbial transmission. Gaining baseline knowledge of the skin-associated microbiome contributes to our knowledge of the natural world and preliminary delineation of ecological relationships between host, microbe, and environment provides an example of the need for continued research in this area which has the potential to broadly inform conservation efforts for amphibians worldwide.

INTRODUCTION

Biodiversity is a cornerstone of ecosystem function. Despite advances in measuring global biodiversity, diversity among microbes remains a poorly understood frontier. All macroscopic organisms serve as host to a tremendous diversity of microscopic organisms on and inside their bodies, collectively known as their microbiome. The microbiome has recently gained notoriety as an important contributor to host health and well-being (1–3). Truly characterizing the microbiome is an area of research only made possible by advances in culture-independent sequencing methods. Techniques such as high-throughput sequencing allow for a more comprehensive understanding of microbial community composition and diversity, as only 0.001%–15% of bacteria can be cultured in a given system, although cultured microbes constituted the most dominant taxa in amphibian skin communities (4, 5). These types of techniques allow us to characterize novel microbiomes, determine microbe-host interactions, and observe microbial transmission. Each are important factors for delineating microbial ecological effects in a system.

Amphibians are no exception to the complex connectedness that all multicellular organisms have with their microbiome. The amphibian skin microbiome is influenced by a myriad of factors including genetics (6), life history (7, 8), behavior (9), physiology (10), environment (11), and exposure to introduced elements (12). Combinations of these factors can influence the microbiome simultaneously, making it challenging to pinpoint sources of microbial disturbance and determine applicable solutions (13). Commonalities have been found among the microbiomes of amphibian skin that can help elucidate how microbes become established on amphibian hosts. For instance, the cutaneous microbiota

of amphibians have been found to be driven by selection processes, including host-species specific selection (6, 8, 14) and immune selection (15, 16). One study found host ecology to be a driving factor in the amphibian cutaneous microbiome (7), suggesting that environmental microbial availability and diversity play a large role in establishing the amphibian skin microbiome. However, there is still no clear answer to the question how most organisms first acquire their microbiota. Is it more how organisms are born or their environment that has a larger impact on their microbial community composition? The extent to which environmental factors contribute to the microbiome are yet to be fully explored and little research has been done delineating between environmental and parental influences.

This study aims to address the fundamental questions of how and where amphibian larvae (tadpoles) acquire their skin microbiome by taking advantage of a specialized breeding strategy utilized by a group of Southeast Asian tree frogs in the genus *Polypedates* (Anura: Rhacophoridae). During successful mating events, adult Rhacophorid frogs produce a foam nest that is attached to vegetation overhanging a body of water (Figure 1). The nest is made from secretions that the mating pair whips into a moist foam in which they simultaneously deposit externally fertilized eggs. Tadpoles undergo early stages of development within the safety of the nest and then exit the nest where they drop into the water below as free-swimming larvae to continue the metamorphic process.

The first aim of this study is to characterize the microbial taxa present on the skin of these frogs, as the skin microbiota have not previously been characterized from this genus. These will also be the first amphibian skin microbiome characterizations from the

island of Borneo in Southeast Asia. Secondly, we aim to provide preliminary data on comparing modes of microbial transmission to determine the influence of parentally versus environmentally derived microbiota on the tadpole skin microbiome. We predict that the nest, in addition to protection, plays a critical role in the establishment of the tadpole microbiome and anticipate that these first microbial colonists will remain on the tadpole skin after the tadpole leaves the nest. Preliminary data regarding microbial transfer will allow for insight into the need for continued research into modes of microbial transfer within this specialized system.

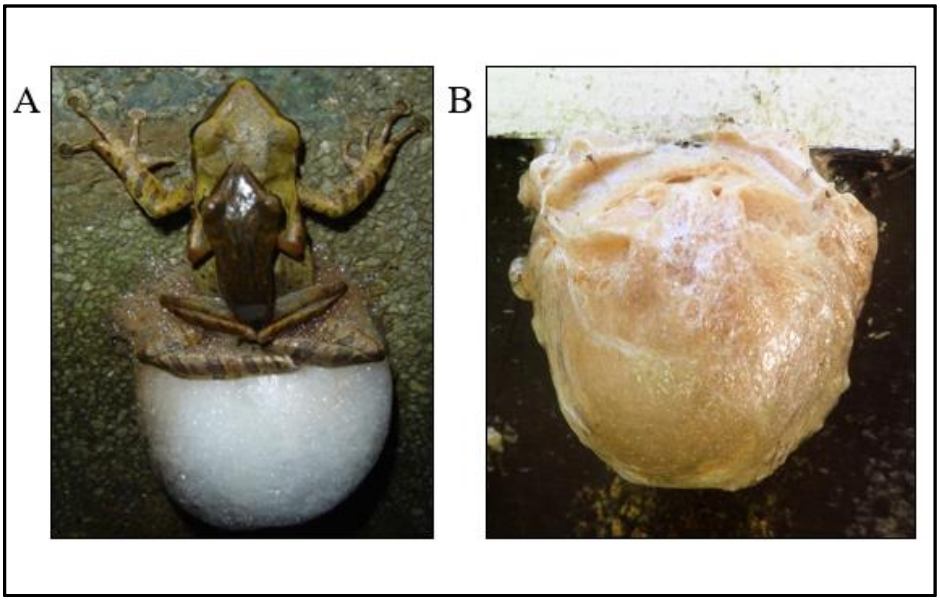


Figure 1. (A) Images of adult *Polypedates leucomystax* in amplexus forming a foam nest on the side of a concrete basin, and (B) a day-old foam nest left by a pair of *P. otilophus* adults on the side of a building at Kuala Belalong Field Studies Centre, Brunei.

Amphibian Declines

Globally, amphibians are in crisis. Populations declines have escalated to include 42% of all described amphibians (17). The rate of amphibian population declines surpasses those of birds and mammals, making them one of the fastest disappearing vertebrate groups on the planet (18). The factors contributing to amphibian declines are complex and intricate (19), however, amphibian declines and extinctions can be linked to two major factors; anthropogenically induced habitat loss and the effects of chytridiomycosis, a pathogenic fungal disease caused by the fungi *Batrachochytrium dendrobatidis* and *B. salamandrivorans*. The astounding loss of amphibian biodiversity caused by these two factors alone has severe implications for ecosystem function and viability. Most amphibians are considered to be indicator organisms, species that serve as a reference for the health of an ecosystem, and the propensity of amphibian declines demonstrates a serious issue in global ecosystem health and function (20, 21). Additionally, with this loss of biodiversity, we not only lose many charismatic and interesting species, but we lose adaptations, physiological specializations, uncharacterized species, and specialized microbial-hosts that are central to further understanding the natural world.

The shift in human lifestyle from hunter-gatherers to large, sedentary societies has reshaped the planet, most notably through deforestation of land for urban development. The human population is currently estimated to be over 7 billion and approximately 75% of Earth's surface shows evidence of human influenced land alteration (22). Habitat change in the form of fragmentation and deforestation typically leaves only a meager proportion of previous biodiversity in its wake (23). Amphibians are particularly

susceptible to anthropogenically driven environmental change due to their biphasic life cycle and semi-permeable skin. Whereas some amphibians thrive as human commensals (24), many species are in decline because of an inability to readily adapt to environmental changes.

A second major factor contributing to amphibian population declines is the pathogenic fungal disease, chytridiomycosis, caused by the fungi, *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*). *Bd* was first officially recognized as a threat to amphibians in the 1990's after having caused decades of ambiguous amphibian deaths in Australia and Central America and to date has caused one of the largest decreases of biodiversity in current history (25, 26). The globalized spread of *Bd*, thanks to human movement and trade, means that *Bd* now affects amphibians in 56 countries (27), the hardest hit regions being Australia (25), Central America (28), South America (29, 30), the Caribbean islands (31, 32), the North American Sierra Nevada (33), and the Iberian Peninsula (34). *Bsal*, the fungal pathogen affecting salamander populations, was more recently introduced to Europe and found to be causing massive declines there since 2010 (35, 36). *Bd* operates by disrupting electrolyte transport across the epidermis, causing reduction of sodium and potassium concentrations, culminating in cardiac arrest and mortality of the host (37–39). *Bsal* causes ulcerative skin lesions to form, resulting in infection and a disruption of cutaneous processes leading to mortality (40). Amphibian population declines due to chytridiomycosis have not yet been detected in Asia and Southeast Asia, where the chytrid fungus has been found to have originated (36). Co-evolution with the fungi may have led to Asian amphibians possessing natural defenses that stave off the disease

effects seen in naïve populations lacking this natural resistance (36, 41). It is therefore critical to characterize amphibian microbiomes from Asia to serve as a baseline in future chytridiomycosis research. We anticipate that the insights and understandings gained from this geographic region will facilitate the development of effective conservation efforts across Asia and, hopefully, other regions across the globe.

The Role of Protective Cutaneous Bacteria on Amphibian Hosts

Amphibian skin requires special consideration due to its semi-permeability that requires a constant mucosal covering. Cutaneous microbiota that inhabit this medium are recognized as affecting host health, disease severity, and adaptation to biotic and abiotic factors (2, 42, 43). Exploration of the amphibian skin microbiome has revealed the application of exploiting microbe-host interactions in the development of new conservation techniques, such as probiotics that inhibit disease effects (43–46). The term "probiotics" encompasses microorganisms ingested or applied in order to confer health benefits. Since the microbiome has been found to be a key factor in amphibian disease mitigation, research into the amphibian skin microbiome has emerged as a topic of favorable examination.

In one such study, Harris et al. (2009) showed that in specific cases probiotics such as *Janthinobacterium lividum*, an antifungal bacterium, can compete with the chytrid fungus to mitigate its effects on host amphibians. Subsequently, Muletz et al. (2012) demonstrated that application of *J. lividum* in the field conferred a protective

effect against *Bd* to certain North American amphibian hosts. However, whereas *J. lividum* is an effective probiotic for certain North American amphibians, Becker et al. (2011) found it ineffective in populations of the Panamanian Golden Frog, *Atelopus zeteki*, a species now extirpated in the wild. Host species seems to influence the probiotic strategy effectiveness, which may be due to immune response (15, 47, 48), or more likely, a combination of factors, including host ecology (7, 49). Interestingly, *A. zeteki* frogs bred in survival assurance colonies seem to maintain a core microbiome shared with their wild ancestors, but have experienced shifts in their overall composition of their skin microbiome (50). This may have implications for these frogs if they are ever to be released back into the wild. Therefore, there is still a strong need to continue baseline characterization research in order to inform probiotic conservation strategies based on host form and function (51).

Amphibians; anurans in particular, undergo dramatic changes in morphology and physiology during the developmental stages of their biphasic lifecycle. It is generally understood that changes in the microbiome accompany major developmental changes (8, 10, 52). Few studies, however, have investigated the amphibian skin microbiome relative to changes in the organism during development and metamorphosis — an important aspect of microbiome characterization. The shift from tadpole to metamorph represents the largest shift in physiology and immune system function (53), and results in the highest instances of mortality due to chytridiomycosis in some species (34). Higher rates of post-metamorphic mortality are due, in part, to the amphibian skin transitioning to a more keratinized state post-metamorphosis so the organism can adapt to life on land. Chytrid fungi thrive in the keratinized skin (10). In one interesting study on probiotic

strategies across development, Davis et. al. (2017) noted the persistence of probiotic microbiota through development in the midwife toad, *Alytes obstetricans*, when administered at the tadpole stage. The ability of probiotics to persist through life stages suggests that larval amphibian stage-specific probiotic strategies may be important in future conservation efforts. However, more research is required to fully understand the changes to skin microbiota through development and the affect these changes have on adult health. Knutie et al. (2017) demonstrated an increased parasite load in adults associated with gut-microbiome disruption at the tadpole stage. This evidence lends to the importance of early stage microbial colonization by "good" microbes, but also begs the question; **How do tadpoles acquire their microbiome to begin with? Is the tadpole skin microbiome influenced more by initial parental contribution or the environment in which they occur?** This study aims to address aspects of these larger questions in order to help inform amphibian conservation.

Modes of Microbial Transmission

In the 4.54 billion years that the Earth has existed, microbes reigned supreme approximately 1.1 billion years before the first multicellular organisms began to evolve (54). This evolutionary time scale allowed for great diversification and the evolution of complex organismal relationships between microbial communities and hosts. These relationships can be mutualistic, symbiotic, commensal, or pathogenic in nature (55–57) and can shift depending on interaction dynamics and microbial transfer (10). For example, natural microbiomes can shift into dysbiosis, or microbial imbalance, in amphibians infested with chytrid fungus and without natural defenses (40, 58). Microbial

transmission can occur in a variety of ways including vertical transmission (parent to offspring), direct horizontal transmission (host to host), indirect horizontal transmission (host to environment to another host), and environmental transmission (environment to host) (59–61). These modes of transmission, and/or a combination thereof, play a major role in host health and relative adaptability (62, 63). Few studies, however, have directly compared modes of transmission and their influence on the microbiome (8, 60, 61). Therefore, this study aims to utilize the specialized breeding strategy of three species of Southeast Asian tree frogs (*Polypedates leucomystax, P. macrotics, and P. otilophus*) that employ a unique foam nest in which their eggs and larvae are isolated from the environment during the earliest developmental stages. This offers a rare opportunity to more easily compare microbial transmission through both vertical and environmental modes (Figure 2).

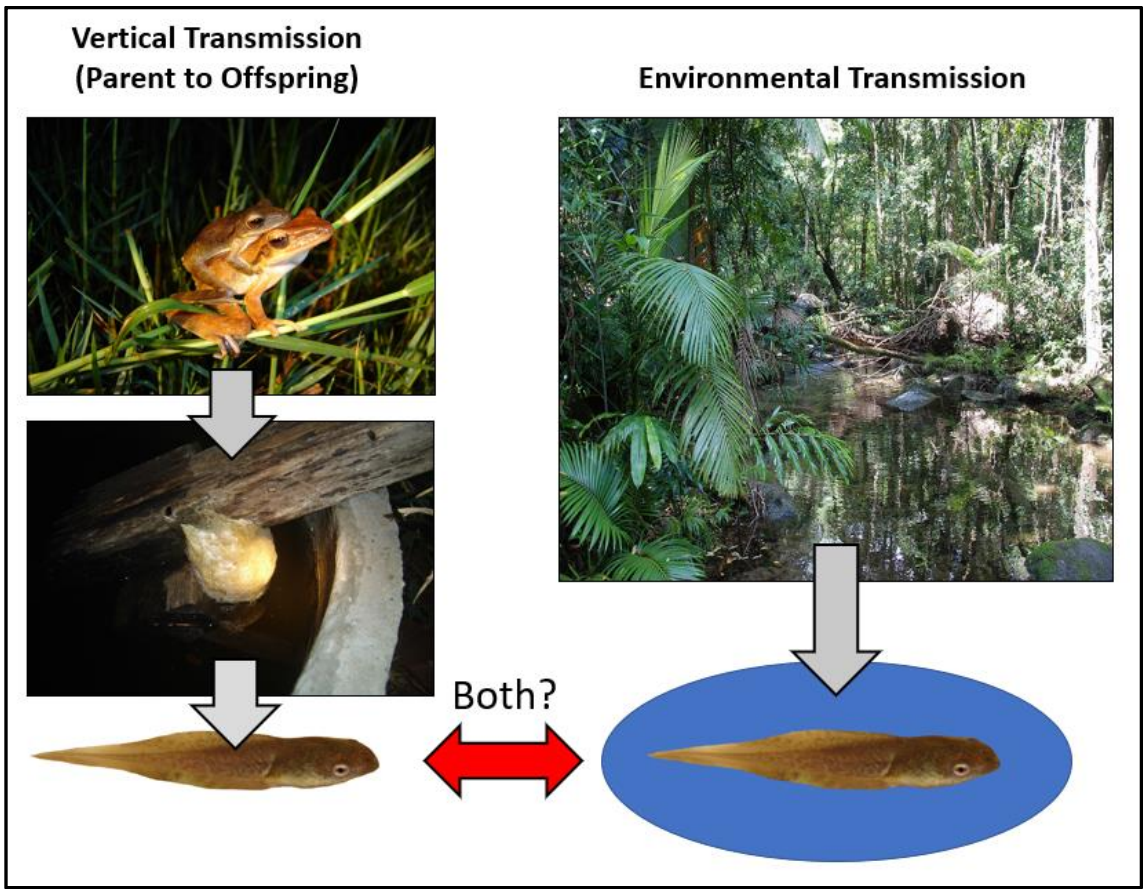


Figure 2. Modes of skin-associated microbiome transmission compared in this study.

16S Amplicon Sequencing

Technology has advanced dramatically since the development of nucleotide sequencing in the 1970's, and demand for methods to deal with massive quantities of genetic data in an accessible way has risen in parallel. Newer high-throughput sequencing methods have the ability to produce sequence reads for multiple samples in one reaction (i.e., massive parallel sequencing). This results in faster and more comprehensive characterization of complex microbial communities (previous culture-based methods yielded a maximum of 15% identification, although most dominant amphibian skin microbiota could be cultured (4, 5)) and their metagenomes (the genomes of microbial populations present in a sample) (64). The ability to sequence multiple samples simultaneously has significantly reduced the cost associated with genetic sequencing, nevertheless, the costs involved remains high and are an important consideration when conducting this type of research (65). Focusing on the 16S region of the ribosomal RNA has been proven effective at delineating microbial taxa due to its universal utility, informative content, and highly conserved nature (66). This standard is now widely used in microbial studies thanks to the expansion of technological and analytical techniques (67–69). Specific drawbacks to this method were noted by Janda and Abbott (2007), including difficulty of accurate taxonomic identification to the species level and nomenclature concerns regarding 16S sequencing databases. However, as this study is exploratory in nature and does not require fine scale taxonomic resolution, we use this method in this study to remain consistent with current amphibian research (6–8, 71, 72).

The Host: Genus *Polypedates*

The family Rhacophoridae comprises 19 genera and 419 species of Asian frogs (https://amphibiaweb.org). Among them, tree frogs of the genus *Polypedates*, have evolved a specialized behavior of depositing foam nest containing fertilized eggs on structures (typically, vegetation) overhanging a body of water (73–75). Tadpoles hatch from eggs within the protection of the foam, and upon emergence from the nest, drop into the water below and continue the metamorphic process (76). The foam is advantageous in that it helps protect the eggs and early tadpoles from predators (77). Kabisch et. al. (1998) found the chemical composition of the *P. leucomystax* foam nest to be composed of 93% protein and 7% sugars (78). This high protein structural content allows nests to remain intact up to 1 month offering protection to the offspring inside (79). *Polypedates leucomystax, P. macrotis, and P. otilophus* were chosen as model systems based on their reproductive mode, year-round breeding regimen (80), and availability at the study site in Brunei (81).

Implications for Conservation

Borneo, the world's third largest island and global biodiversity hotspot, is replete with lush jungles and enigmatic species, such as the orangutan. Unfortunately, Borneo is also tremendously affected by slash-and-burn deforestation practices that eliminate native forests for the expansion of the palm oil industry (82). Borneo's biodiversity is disappearing at an expedited rate due to anthropogenic influences and is consequently recognized as an area of exceptional conservation concern (83). The island of Borneo

comprises three different countries (Figure 3). The Indonesian state of Kalimantan occupies the majority of the island. The Malaysian states of Sabah and Sarawak lie in the northern portion of the island and surround Brunei, an independent sultanate and the location of this study (Figure 3). The field study site, located in the Temburong District of Brunei at the Kuala Belalong Field Studies Centre (KBFSC), is surrounded by the protected forests of Ulu Temburong National Park. This region is an important natural area rich with biodiversity (81), an increasingly rare habitat on the island due to the encroachment of urbanization and the consummation of rainforest land for plantation agriculture. Characterizing novel microbiomes from amphibians in this location is vital as no such research has previously been conducted for this area, endemic amphibian species are prolific on the island, and impeding anthropogenic encroachments threaten this pristine habitat.

Figure 3. Map showing the location of Brunei within the context of Southeast Asia. The inset in the upper right-hand corner shows Brunei's (dark green) location on the island of Borneo and is a magnification of the red outlined area on the foreground map. The yellow dot denotes the field site location.

MATERIALS AND METHODS

Study Site

Field studies were conducted between May and July 2017 from locations around the Kuala Belalong Field Studies Centre (KBFSC) in Ulu Temburong National Park, Brunei (Figure 4). KBFSC is situated within a tropical mixed-Dipterocarp rainforest and is home to at least 53 different frog species (81). The region surrounding KBFSC consists of steep ridges and valleys through which two large, permanent rivers (Sungai Temburong and Sungai Belalong) and their tributaries flow. Maximum elevation at KBFSC is 60m ASL (84). Average annual rainfall ranges between 250–400 cm/year (85). Samples were collected from two sites within the park. The first is a permanent pond located in disturbed secondary forest across the Temburong river from Ulu Ulu Resort, upriver from KBFSC (Figure 4A). This pond was formed when earth was moved to create a road to run powerlines to the resort. The pond measures approximately 5m wide, 9m long, and 1.5m at maximum depth with dark, tannin rich water and a substrate of fine sediment covered by dense vegetative detritus. Woody shrubs, herbaceous vegetation, vines, and trees surround the pond. The forest canopy reaches 4m high over the pond and is mostly closed with a few open patches where trees have fallen. During the collection period, six species of amphibians were observed in or around the pond, including; *Polypedates macrotis, P. otilophus, Limnonectes kuhlii, L. leporinus, Rhacophorus pardalis,* and *Occidozyga laevis*. More frog species were heard calling, but not observed. The second site, a 1.5m diameter concrete basin with 0.5m maximum water depth and approximately 10cm of fine silt covered by a layer of detritus at the

bottom, is located on the grounds of KBFSC and is part of a water drainage system used to divert water away from buildings (Figure 4B). The canopy over the basin is open. Vegetation reaching 1m high and overhang part of the water is present on one side of the basin and consists various of plants, including pandan (*Pandanus amaryllifolius*). During the collection period *P. leucomystax* and *P. otiolophus* species were observed in or around the basin.

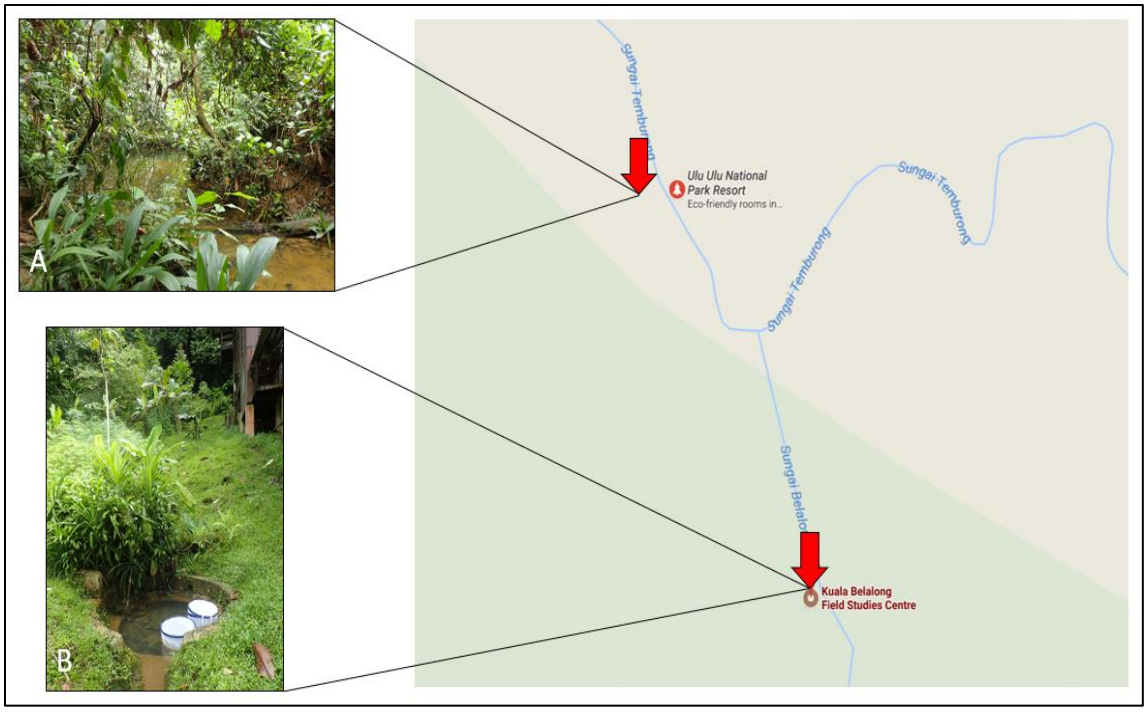


Figure 4. Map of site locations in Ulu Temburong National Park, Brunei. The top image is of the secondary forest pond site (A) and the bottom image is of the KBFSC basin site (B) while containing two of the in-field enclosures with tadpoles.

Sample Collection

Adults of three species were selected for sampling: *Polypedates leucomystax* (Four-lined Treefrog), *P. macrotis* (Dark-eared Treefrog), and *P. otilophus* (File-eared Treefrog), (Figure 5). These three species occur syntopically in Ulu Temburong National Park. Collection of mating pairs was completed during nocturnal surveys where collectors sat and waited for frogs of the focal species to engage in amplexus. Surveys were conducted from 18:00–23:00 hrs. Pairs in amplexus were captured by hand and placed into a clean plastic bag for transport to the lab at KBFSC. All pairs but one were captured and handled by investigators using sterile nitrile gloves. One pair of frogs was handled with ungloved hands but was included in this study to ensure triplicate samples of nests and associated variables. Once back at the lab pairs were placed in plastic terraria (approximately 30 cm x 25 cm x 50 cm) sterilized with 100% ethanol. The first pair placed in a bare terrarium would not engage in amplexus, so to facilitate the mating process, approximately 2 cm of water from the concrete basin and one to two large leaves from the same location were added to the terrarium to facilitate breeding. As the first pair was found to forego amplexus without these naturalized conditions, the same process was repeated for subsequent pairs for consistency.

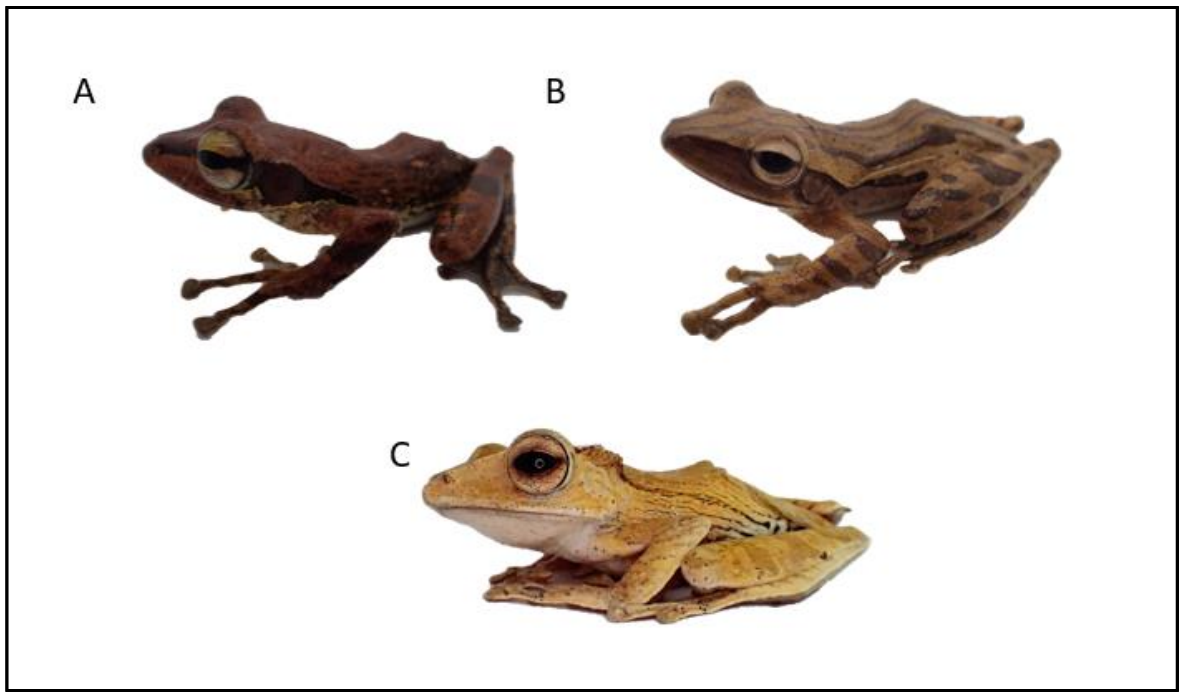


Figure 5. Images of the three species of *Polypedates* adults sampled. (A) *P. macrotis*, Dark-earred Treefrog; (B) *P. leucomystax*, Four-lined Treefrog; (C) *P. otilophus*, File-earred Treefrog. Photos by David S. McLeod.

Once pairs had completed nest deposition, each individual was removed from the terraria using new sterile nitrile gloves. Individuals were rinsed with 100ml of distilled water before swabbing to ensure sampling of amphibian skin-associated microbes rather than transient microbes or environmental material (14, 86, 87). New bottles of Suci brand distilled drinking water (330ml bottles, Suci Mas Company, Bandar Seri Begawan, Brunei Darussalam) were used for each individual specimen to decrease inter-sample contamination. Each specimen was swabbed for 15 seconds at each of three different body locations (cloaca, dorsum, and venter) using different sterile rayon swabs (MW113, Medical Wire Equipment & Co. Ltd., Corsham, UK) (6). To ensure that individuals were not re-sampled and to provide whole voucher specimens for additional studies, specimens were euthanized in a dilute solution of MS222, fixed in 10% neutral-buffered formalin, and later transferred to 70% EtOH. Liver tissue samples taken prior to fixation were stored in RNAlater. The use of animals for this study was approved by the Institutional Animal Use and Care Committee of James Madison University (A15-15) and was completed with permission from the University of Brunei Darussalam (UBD/AVC-RI/1.21.1[a]).

Anecdotally, in addition to the three pairs discussed above (Figure 6), another pair of *P. leucomystax* was captured by other field scientists as part of another study. The male and female were placed in separate plastic bags and the female produced a foam nest in the bag by herself, not having been in contact with the male during the formation of the nest. These adults, as well as the resulting infertile nest, were sampled and included in sequencing to allow for the comparison of nests made by pairs versus that made by a single female individual. The infertile foam nest was sampled approximately

six hours after formation of the nest by swabbing for 15 seconds, along with both of the adults. Adults were sampled using the same protocol as all other adult pairs and the nest was sampled in triplicate. Microbial community characterization of these samples is included in the appendix but not included in the main analyses for clarification.

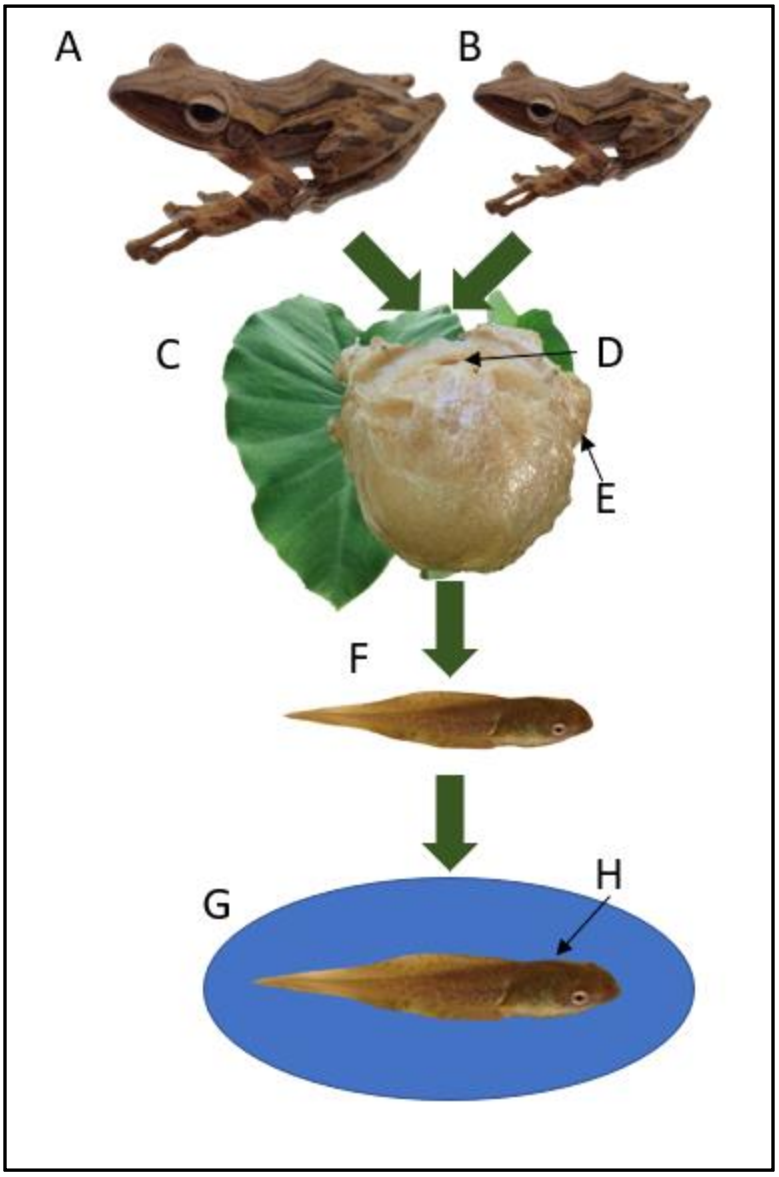


Figure 6. Visual representation of all variables sampled to characterize the microbial communities across a developmental and environmental gradient. (A) Adult female and (B) adult male individuals were sampled body location. (C) Leaves were the attachment point for foam nests, which were sampled (D) inside and (E) outside for comparison. (F) Tadpoles extracted from the nest were compared to (G) water samples and (H) tadpoles after having one week of environmental interaction in a pond in-field enclosure. Images taken by David S. McLeod.

Within the first few days following deposition, the exposed surfaces of the foam nest acquire a crust-like exterior that seems to protect the tadpoles and prevents desiccation [pers. obs.]. Approximately one week after collection, all contents of the terraria were sampled including nests, leaves, water, and tadpoles from inside the nest were swabbed. This is when movement was first observed in the nest and/or the first tadpoles had moved from the nest into the water within the terraria. To better understand nest bacterial biodiversity and the relationship between nest, parental, and environmental microbes, both exterior and interior aspects of the nests were swabbed. Exterior surfaces were sampled by rubbing a sterile swab over the surface of the nest for 15 seconds. The interior was sampled by gently creating an opening indentation with a gloved finger and inserting a sterile swab, moving it around within the nest for 15 seconds. All foam nest samples were completed in triplicate. Second and third interior foam nest sampling swabs were inserted via the same opening to maintain nest integrity.

Tadpoles from each clutch were sampled at two different time points in development, once before emerging from the foam nest and again after a period of exposure to the environment outside of the nest. These time points were chosen to facilitate determination of cutaneous microbial community shifts of tadpoles before and after environmental interaction. Several tadpoles from the foam nest were extracted after the nest had been swabbed and the inside was made accessible via a small opening created for interior nest sampling. This was done using a sterilized 15 mL plastic pipette cut to create a large enough opening for tadpoles to pass through. Several tadpoles were deposited into a single sterile, plastic container where they were rinsed with 50 mL of

distilled water. Tadpoles were rinsed together because their small size prohibited rinsing each one individually. Three were randomly chosen for sampling from the container. Each individual was placed on a newly gloved hand and swabbed all over, ventrally and dorsally, for 15 seconds, equating to roughly 5–7 strokes on each side (60) using a sterile swab.

To determine the microbial diversity of the environment directly communicating with the nests and tadpoles, samples were obtained from the leaves that nests were attached to and water samples from both inside terraria and from inside in-field enclosures. Leaves were sampled directly after foam nest sampling (approximately one week after nest deposition) by swabbing the leaf surface for 15 seconds. Terraria water samples were obtained at the same time by running a swab through the water for 15 seconds. Enclosure water samples were obtained after tadpoles had acclimated in in-field enclosures for one week, before dip netting with a sterilized aquarium dip net to remove tadpoles for sampling, by inserting a swab approximately 10cm into the water column within the enclosure and moving at this depth for 15 seconds (8). All environmental samples were completed in triplicate.

Once all of the remaining tadpoles from the nests hatched, they were placed in in-field enclosures consisting of 45.72cm x 66.04cm mesh laundry hampers with mesh, zippered lids (Collapsible Laundry Hamper, Whitmor Inc., Southaven, MS, USA) inside the concrete basin at KBFSC. Each enclosure contained larvae from a single clutch and allowed for maximum interaction with the environment while containing the tadpoles and protecting from predation and contact with other conspecifics living in the basin. After one week, samples from tadpoles and water inside the enclosure were collected.

Tadpoles were removed from enclosures via a sterilized dipnet, placed in a sterile plastic container, and taken back to the lab where the same protocol was followed as for the tadpoles extracted from the nest.

For all sampling, swabs were immediately placed in sterile (autoclaved) 1.5ml Nalgene cryotubes (Thermo Fisher Scientific, USA), stored inside vacuum insulated canisters (Rambler 64oz & 36oz, Yeti Coolers LLC, Austin Texas, USA) and placed in a -20°C freezer. Samples were maintained in this manner for 23 days until their export to Brunei's capital, Bandar Seri Begawan, where they were kept at -20°C constantly for 24 days. During the approximately 30 hours of travel during export to the United States, samples were able to be maintained in a frozen state and subsequently transferred to a -80°C freezer at James Madison University until processing.

Sample Processing

Amplicon sequencing of the 16s rRNA gene was used to determine bacterial community structure for all amphibian and environmental variables. DNA was extracted using the DNeasy PowerSoil Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The V4 region of the 16S rRNA gene was PCR-amplified with barcoded primers following the 16S Illumina Amplicon Protocol standard for the Earth Microbiome Project (515f/806r, press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s/). Each 25 μL PCR contained: 6.5 μL molecular grade PCR water, 12.5 μL 5 Prime Hot Master Mix, 0.5 μL each of the forward and reverse primers, and 5 μL genomic DNA. PCR conditions were: denaturation step 3 min at 94°C, amplification

step for 35 cycles for 45 sec at 94°C, annealing for 60 sec at 50°C, extension for 90 sec at 72°C, and a final extension of 10 min at 72°C. Amplified samples were run on a 1% agarose gel to check for amplicons and then cleaned using AMPure XP Beads (Beckman Coulter, Inc., Brea, CA, USA) according to the PCR Clean-Up protocol outlined on page 8 of Illumina's 16S Metagenomic Sequencing Library Preparation guide (15044223 B). Unique dual indices were then attached to each cleaned product in a second PCR step. For this round, each 50 µL PCR contained: 25 µL 5 Prime Hot Mastermix, 5 µL index 1 primer, 5 µL index 2 primer, 10 µL molecular grade PCR water, and 5 µL amplified genomic DNA (see appendix 1 for indexing information. PCR conditions were: denaturation step 3 min at 95°C, ligation for 8 cycles for 30 sec at 95°C, 30 sec at 55°C, 30 sec at 72°C, and a final extension for 5 min at 72°C. These products were checked for integrity on a 1% agarose gel and then quantified using the Qubit dsDNA HS Assay Kit (Thermofisher, Waltham, MA, United States). Equal concentrations of each sample were pooled and the library pool was sequenced on two Illumina MiSeq runs using 2 x 250 paired end technology at the Genomics and Microbiology Research Lab of the North Carolina Museum of Natural Sciences.

Sequence Processing

Sequence reads were quality filtered and processed using the program Quantitative Insights Into Microbial Ecology 2 (vQIIME2-2018.2) (68, 88). Demultiplexed forward reads from two Illumina MiSeq 2x250 platform runs were imported and filtered using the following criteria: minimum PHRED score of 4, a

maximum of three consecutive low-quality PHRED scores observed before truncation, and zero ambiguous base calls (N's) within the sequence. Only forward reads were used for analysis due to the poor quality of reverse reads for both Illumina runs (7, 89, 90). Both runs were analyzed concurrently but not merged to determine run effects. Quality filtered sequences were trimmed to 220bp and clustered into sub-operational taxonomic units (sOTU's) using the Deblur workflow (7, 91), hereafter referred to solely as OTU's. Taxonomy was then assigned by aligning sequences with the Greengenes 13_8 99% database (Naïve Bayes classifier trained on the 515f/806r region) and a phylogenetic tree was built using the fasttree algorithm (92). Sequencing depth per sample ranged from 4,189 to 22,641, equating to a 5-fold increase. Due to this relatively low difference in library sizes we did not rarefy as that would not improve our false discovery rate and might introduce biases (93, 94). The final OTU table was filtered to keep OTUs that had at least two representative sequences and that were detected in at least 2% of samples (N=101) using the phyloseq package in R (v3.5.0) (phyloseq 1.24.0). All subsequent analyses were conducted in R.

Data Analysis

We first completed a general descriptive analysis of the sequence data. This consisted of OTU and distribution summaries across all samples. Then analyses were completed for all amphibian samples across an initial developmental gradient (e.g., adult male and adult female, nest inside and outside) along with comparing amphibian and environmental samples to determine environmental transmission. Across all comparisons, we tested for differences in alpha diversity using Faith's phylogenetic

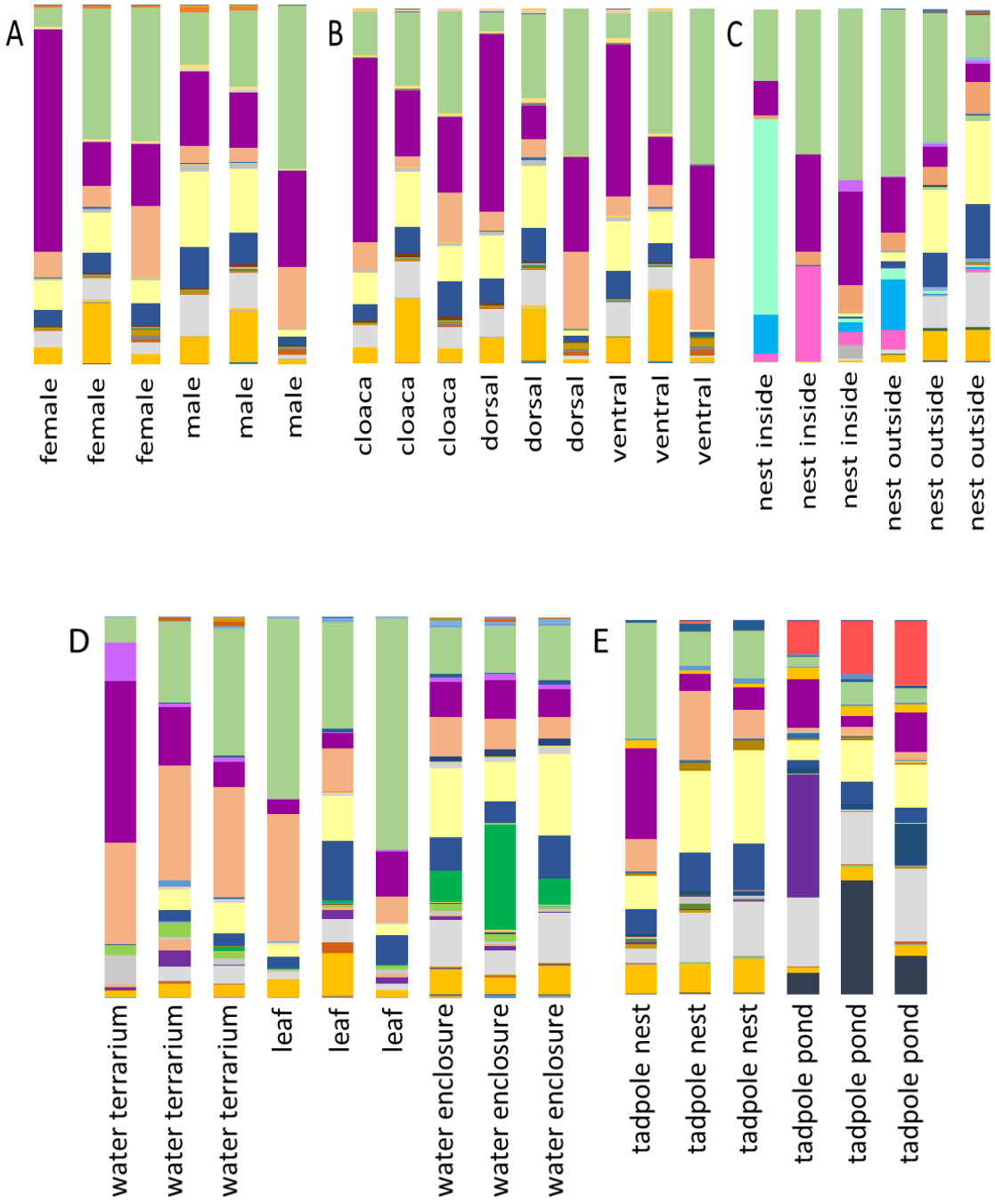
diversity (PD) and species richness (SR). ANOVAs were used to compare both alpha diversity metrics. Beta diversity tests were run on Jaccard and unweighted Unifrac distance matrices to compare presence and absence of microbial taxa as well as a Bray Curtis distance matrix as a comparison of presence/absence and relative abundance of taxa. These matrices were used to test for differences in diversity among variables using permutational multivariate analysis of variance (PERMANOVA) tests at 999 permutations. PERMANOVAs were completed in R (95) using the adonis function in the 'vegan' package (96).

Due to sample size constraints (n=3; one nest per species for *Polypedates leucomystax*, *P. macrotis*, and *P. otilophus*) all nest samples were considered together as congeners and analyzed concurrently. Adults were compared to determine any differences in their respective microbial communities, particularly to delineate differences between sex or body location (dorsal surface, ventral surface, cloaca). When testing for differences in sex, body location was accounted for by using the "strata" argument in the adonis function in R. Results of foam nest analyses were compared by location (inside and outside) as were results across all variables, including; adults (only the cloaca samples were used here and serve as a representation of 'adults' due to no observed differences by body location and sex), foam nests, tadpoles extracted from the nest for sampling (tadpole-nest), tadpoles sampled after one week of interaction with their pond environment (tadpoles-pond), water variables, and leaves. Additionally, tadpoles-nest and tadpoles-pond were compared directly to observe microbial community differences driven by environmental interaction.

RESULTS

Community Overview

Across all samples (n=101) a total of 1,101,191 sequences were analyzed resulting in 2,787 distinct OTU's. Two archaeal phyla and 23 bacterial phyla were found to be present across samples. Proteobacteria constituted the dominant percentage of microbial communities at 57% relative abundance. Other common phyla included Firmicutes (16%), Bacteroidetes (13%), Actinobacteria (5%), Cyanobacteria (2%), and Verrucomicrobia (2%) (Figure 7).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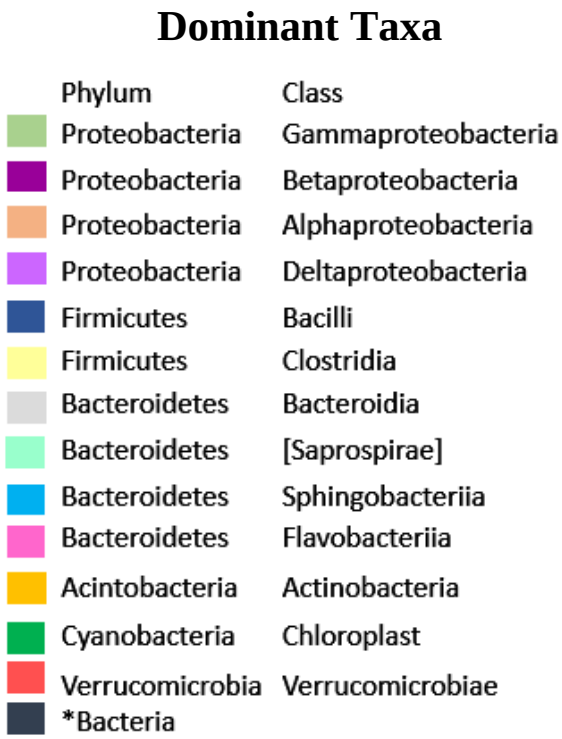


Figure 7. Taxonomic bar plots depicting the bacterial community composition of amphibians and their environment. (A) Adult frogs by sex. (B) Adult frogs by body location sampled. (C) Inside and outside foam nest samples. (D) Environmental variables including the water placed in the terrarium during foam nest formation and water sampled from the in-pond enclosures the tadpoles were kept in during environmental assimilation. (E) Tadpoles extracted directly from the foam nest and tadpoles sampled after one week of environmental interaction. * denotes a Kingdom instead of a Phylum.

Comparison of Adults

Adult sex did not affect skin microbiome richness or composition (female n=3, male n=3). Adult male and female frogs did not differ significantly from one another in alpha diversity (ANOVA; SR, $F = 0.25$, $p = 0.624$; and PD, $F = 0.147$, $p = 0.706$) or beta diversity (PERMANOVA – Jaccard: Pseudo-F = 0.768, $R^2 = 0.045$, $p = 0.823$, **figure 8**; Unweighted Unifrac: Pseudo-F = 0.856, $R^2 = 0.05$, $p = 0.639$; Bray-Curtis: Pseudo-F = 0.787, $R^2 = 0.046$, $p = 0.552$).

Among individual adult frogs, microbiome richness and composition did not differ among body locations (cloaca, dorsal surface, ventral surface) in terms of alpha diversity (ANOVA; SR, $F = 0.255$, $p = 0.777$; and PD, $F = 0.179$, $p = 0.837$) or beta diversity (PERMANOVA – Jaccard: Pseudo-F = 0.733, $R^2 = 0.065$, $p = 0.983$, **figure 9**; Unweighted Unifrac: Pseudo-F = 0.758, $R^2 = 0.067$, $p = 0.959$; Bray-Curtis: Pseudo-F = 0.355, $R^2 = 0.032$, $p = 0.995$).

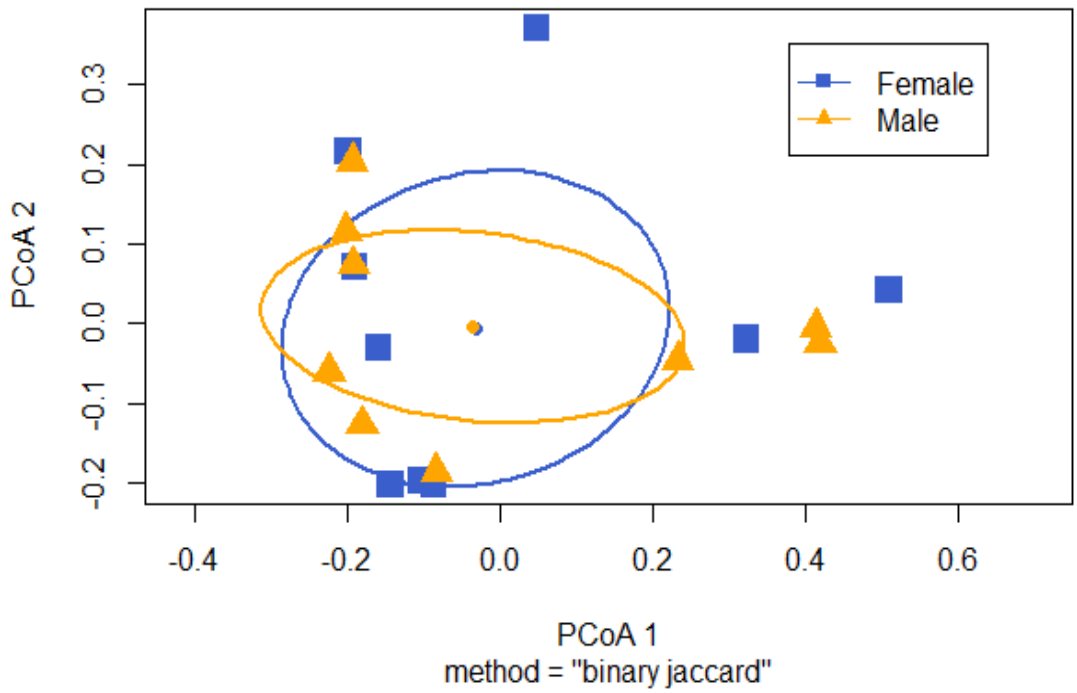


Figure 8. Principle coordinate analysis representing the composition of bacterial communities of adult frogs by sex using a Jaccard distance (presence/absence of taxa). Smaller, centered dots represent the centroid, or mean, of each set of points.

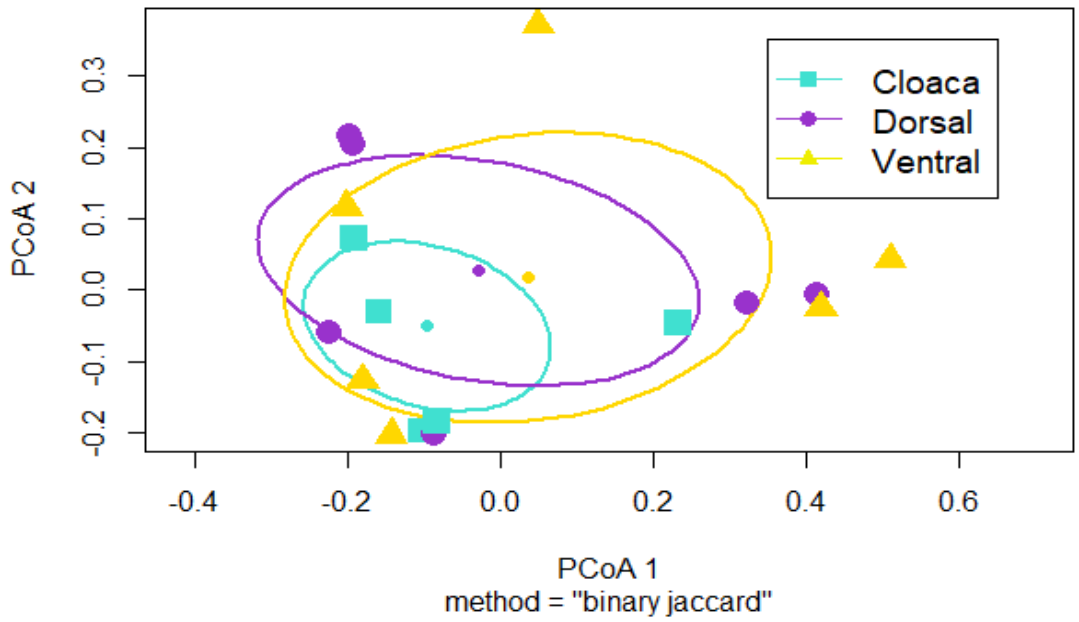


Figure 9. Principle coordinate analysis representing the composition of bacterial communities associated with the various regions of body location sampled on all adult frogs using a Jaccard distance (presence/absence of taxa). Smaller, centered dots represent the centroid, or mean, of each set of points.

Analysis of Foam Nest Bacterial Communities

Microbiome richness and composition differed between the inside and the outside of the foam nest. Alpha diversity differed between locations (species richness: ANOVA, $p = 0.005$, phylogenetic diversity: Wilcox test, $p = 0.003$). Beta diversity comparisons show significant differences in bacterial community taxa and relative abundance (PERMANOVA – Jaccard: Pseudo-F = 4.863, $R^2 = 0.233$, $p = 0.001$, **figure 10**; Unweighted Unifrac: Pseudo-F = 5.206, $R^2 = 0.245$, $p = 0.001$; Bray-Curtis: Pseudo-F = 3.993, $R^2 = 0.199$, $p = 0.002$). Analysis of Similarity confirmed differences within and between sampling locations (ANOSIM, $R = 0.699$, $p = 0.001$). The inside of the foam nest, while demonstrating more variability than the outside nest (Figure 10), clusters together by specific nest (Figure 11).

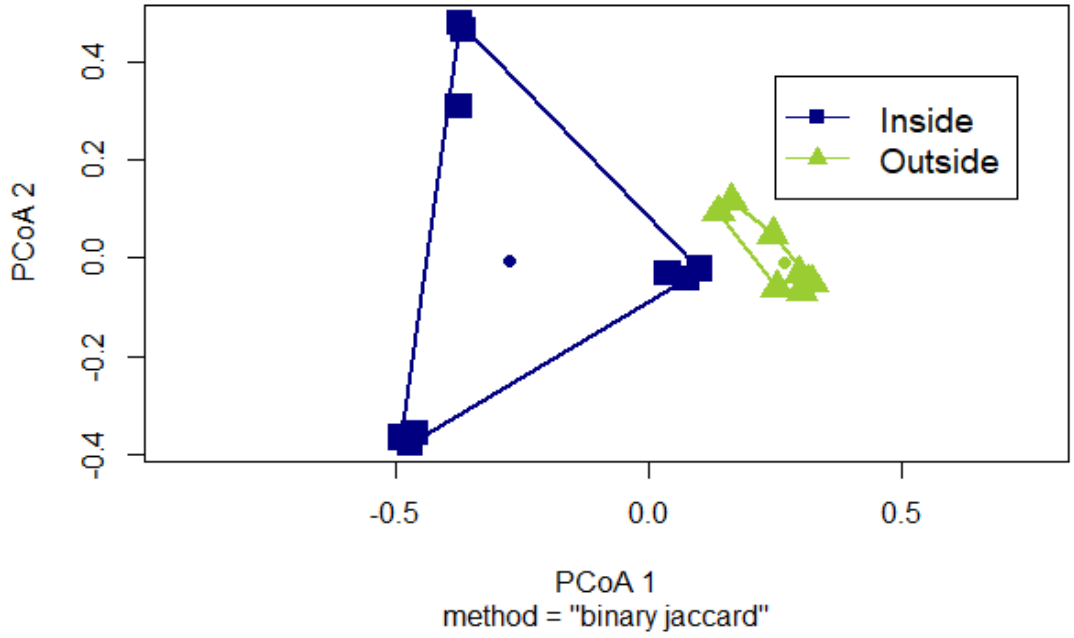


Figure 10. Principle coordinate analysis plot representing presence/absence of microbial communities in the interior and exterior sampling locations of each foam nest. Each individual point represents one of the triplicate samples taken from each nest.

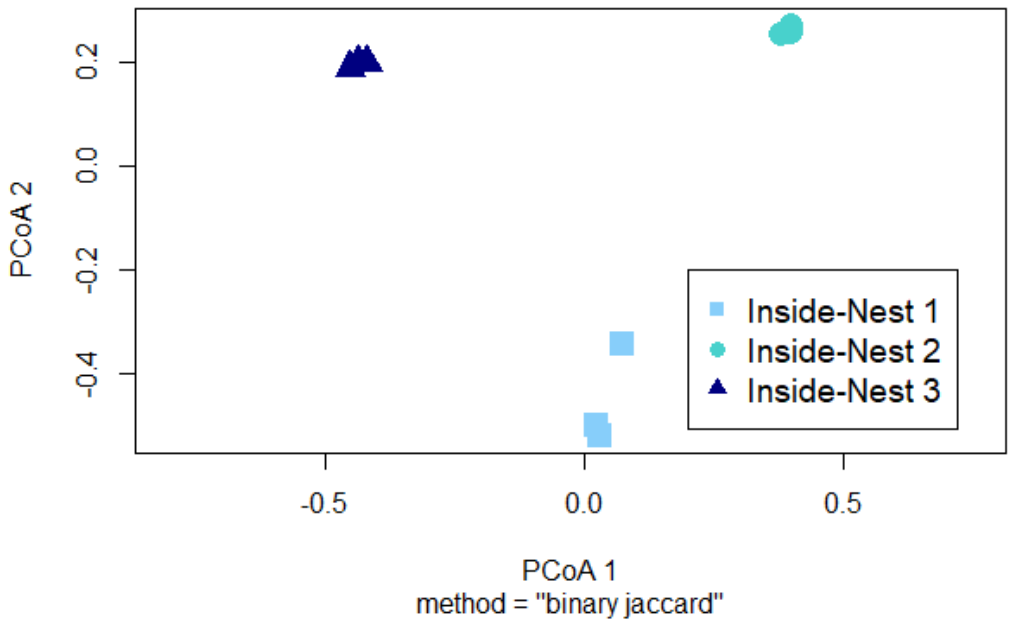


Figure 11. Principle coordinate analysis plot of specific nest interiors sampled representing relative taxonomic structure of nest inside bacterial communities. Each individual point represents one of the triplicate samples taken from each nest.

Amphibian Bacterial Communities Differ from their Environment

The microbiome differed among adults, tadpoles, and their environment.

Pairwise PERMANOVA analyses showed significant differences among life stages and environmental microbial community composition, demonstrated by the Jaccard beta diversity metric (Table 1), however, significant values were present for all comparisons in the table across three beta diversity metrics, Jaccard, Unweighted Unifrac, and Bray Curtis. The only exception existed for the comparison of adult frogs to leaf samples which had a significantly different relative abundance of similar taxa (PERMANOVA – Jaccard: $p>0.05$; Unweighted Unifrac: $p>0.05$; Bray-Curtis: $p<0.05$). Alpha diversity metrics show variability in species richness and phylogenetic diversity across variables (Table 2). Differences in relative abundance and taxonomic presence between communities is presented visually as stacked bar graphs in Figure 7. A visual representation of the relative abundance of microbiota designated to the class taxonomic level show the differences and similarities between individual samples associated with each variable.

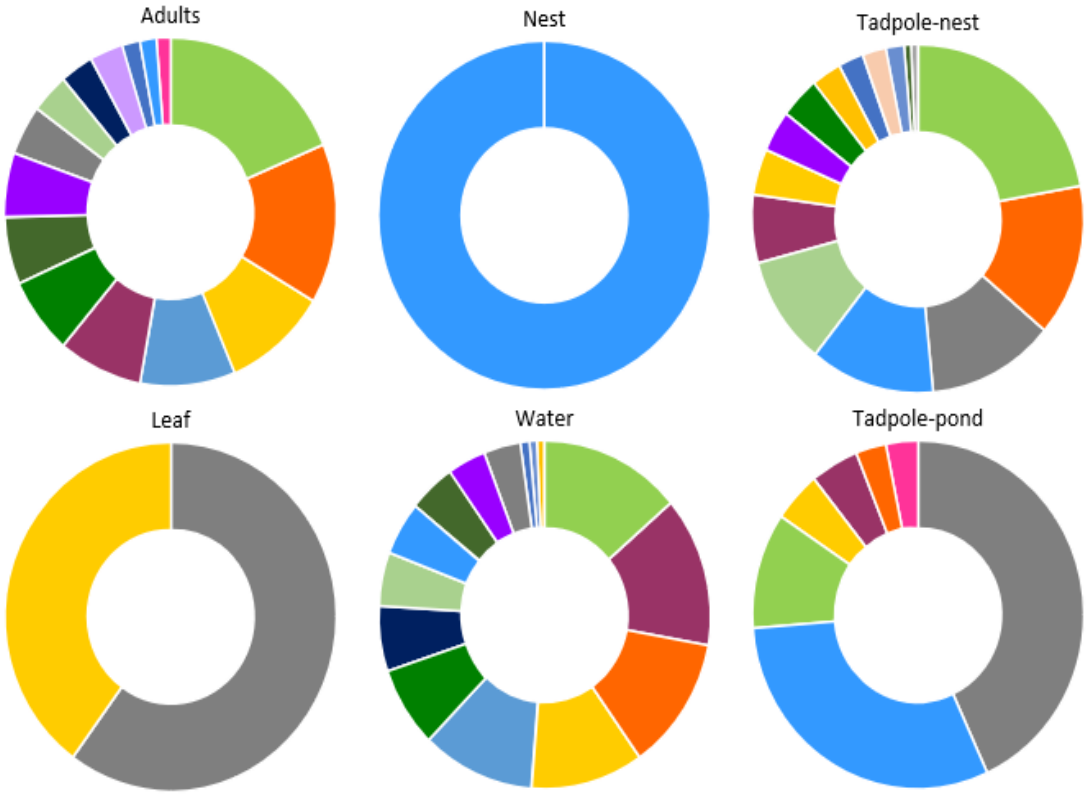
Table 1. Pairwise PERMANOVA results showing significant differences between variables using a Jaccard distance beta diversity metric.

Comparisons		P<0.05
Adult-	tadpole-pond	**0.0023**
	water	**0.0035**
Leaf-	tadpole-nest	**0.0113**
	tadpole-pond	**0.0023**
	water	**0.0023**
Nest-	tadpole-nest	**0.0196**
	tadpole-pond	**0.0035**
	water	**0.0035**
Tadpole-nest-	tadpole-pond	**0.0023**
	water	**0.0023**
Tadpole-pond-	water	**0.0023**

Table 2. Species richness and phylogenetic diversity metrics across amphibian and environmental variables given as mean ± standard error.

Category	Species Richness	Phylogenetic Diversity
Adult	593 ± 51.75	24.99 ± 1.48
Nest	359.16 ± 114.04	16.12 ± 4.11
Tadpole-nest	646.33 ± 58.71	27.34 ± 1.72
Tadpole-pond	598.11 ± 58.71	26.82 ± 1.98
Leaf	400.22 ± 94.33	18.16 ± 3.49
Water-terrarium	402 ± 72.45	19.01 ± 2.66
Water-enclosure	775.66 ± 40.4	32.33 ± 1.22

The core microbiome was examined across all sequences and 15 taxa were found to be present in 85% of samples. In breaking down the core microbiome by variable type, the presence and abundance of microbial taxa can be compared between amphibian and environmental samples. The largest majority of core taxa are in the phylum Firmicutes with the phylum Proteobacteria representing a large portion as well, particularly in the nest and tadpole-pond samples. Foam nest samples contained the most exclusive core microbiome with 99% core composition found to be one taxa, *Acinetobacter guillouiae* (Figure 12).



Phylum	Family	Genus/Species
Actinobacteria	Micrococcaceae	*Arthrobacter*
Bacteroidetes	–	–
Bacteroidetes	[Paraprevotellaceae]	*CF231*
Cyanobacteria	–	–
Firmicutes	Planococcaceae	*Rummeliibacillus*
Firmicutes	Staphylococcaceae	*Staphylococcus*
Firmicutes	Lactobacillaceae	*Lactobacillus*
Firmicutes	Lactobacillaceae	*Lactobacillus reuteri*
Firmicutes	Streptococcaceae	*Streptococcus luteciae*
Firmicutes	–	–
Firmicutes	Ruminococcaceae	–
Firmicutes	Ruminococcaceae	*Faecalibacterium prausnitzii*
Firmicutes	Erysipelotrichaceae	–
Proteobacteria	Moraxellaceae	*Acinetobacter guillouiae*
Tenericutes	–	–

Figure 12. Relative abundance of the core microbiome present on 85% of all samples separated by variable groups. Legend contains relevant taxonomic designations for the core microbiome across samples.

Tadpole Bacterial Community Before and After Environmental Interaction

Tadpoles extracted from the foam nest and those kept in in-field enclosures for one week differed in the structure of their cutaneous microbial communities (PERMANOVA – Jaccard: Pseudo-F = 2.145, R^2 = 0.118, p = 0.001; Unweighted Unifrac: Pseudo-F = 2.580, R^2 = 0.138, p = 0.002). Whereas these results show significant differences between tadpoles in terms of presence/absence of taxa, stacked bar plots (Figure 7, E) and the Bray-Curtis principle coordinate analysis plot (Figure 14) show distinct differences in relative abundance shifts for bacterial taxa after environmental interaction, reinforced by permutational multivariate analysis (PERMANOVA – Bray-Curtis: Pseudo-F = 5.441, R^2 = 0.253, p = 0.001).

As for the tadpole specific core microbiome, 100% of tadpole samples contained 15 bacterial taxa before and after interaction with the environment (Figure 15). Again, the majority are in the phyla Proteobacteria and Firmicutes.

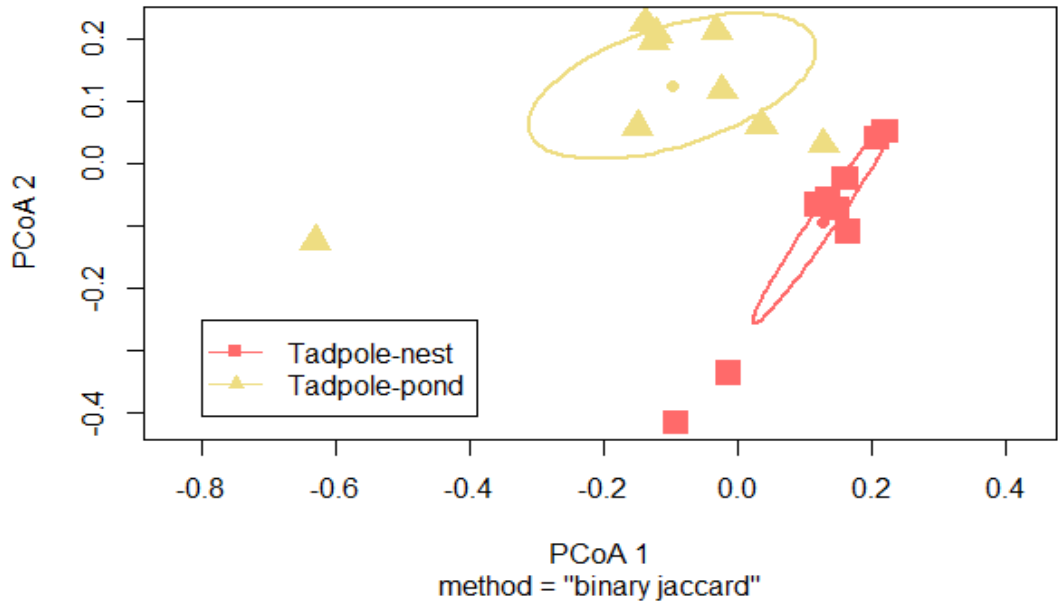


Figure 13. Principle coordinate analysis plot of the tadpole skin microbiome before and after environmental interaction using a Jaccard distance matrix (presence/absence of taxa). Tadpole-nest are those tadpoles extracted from the nest for sampling and Tadpole-pond refers to tadpoles sampled after one week of environmental interaction in an in-field enclosure.

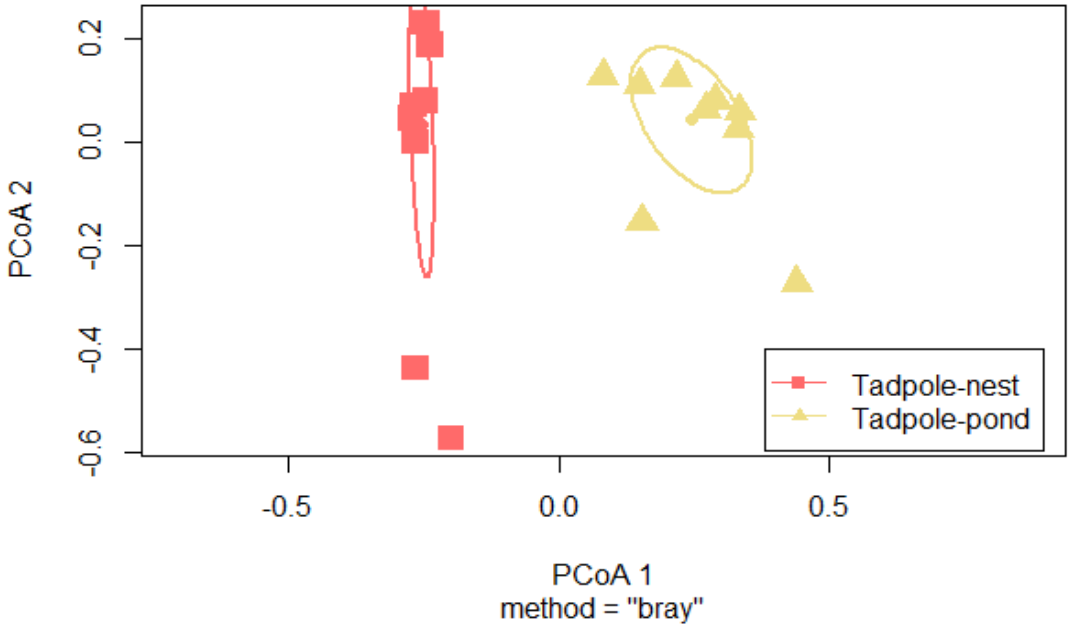


Figure 14. Principle coordinate analysis plot of the tadpole skin microbiome before and after environmental interaction using a Bray-Curtis distance matrix (abundance of taxa). Tadpole-nest are those tadpoles extracted from the nest for sampling and Tadpole-pond refers to tadpoles sampled after one week of environmental interaction in an in-field enclosure.

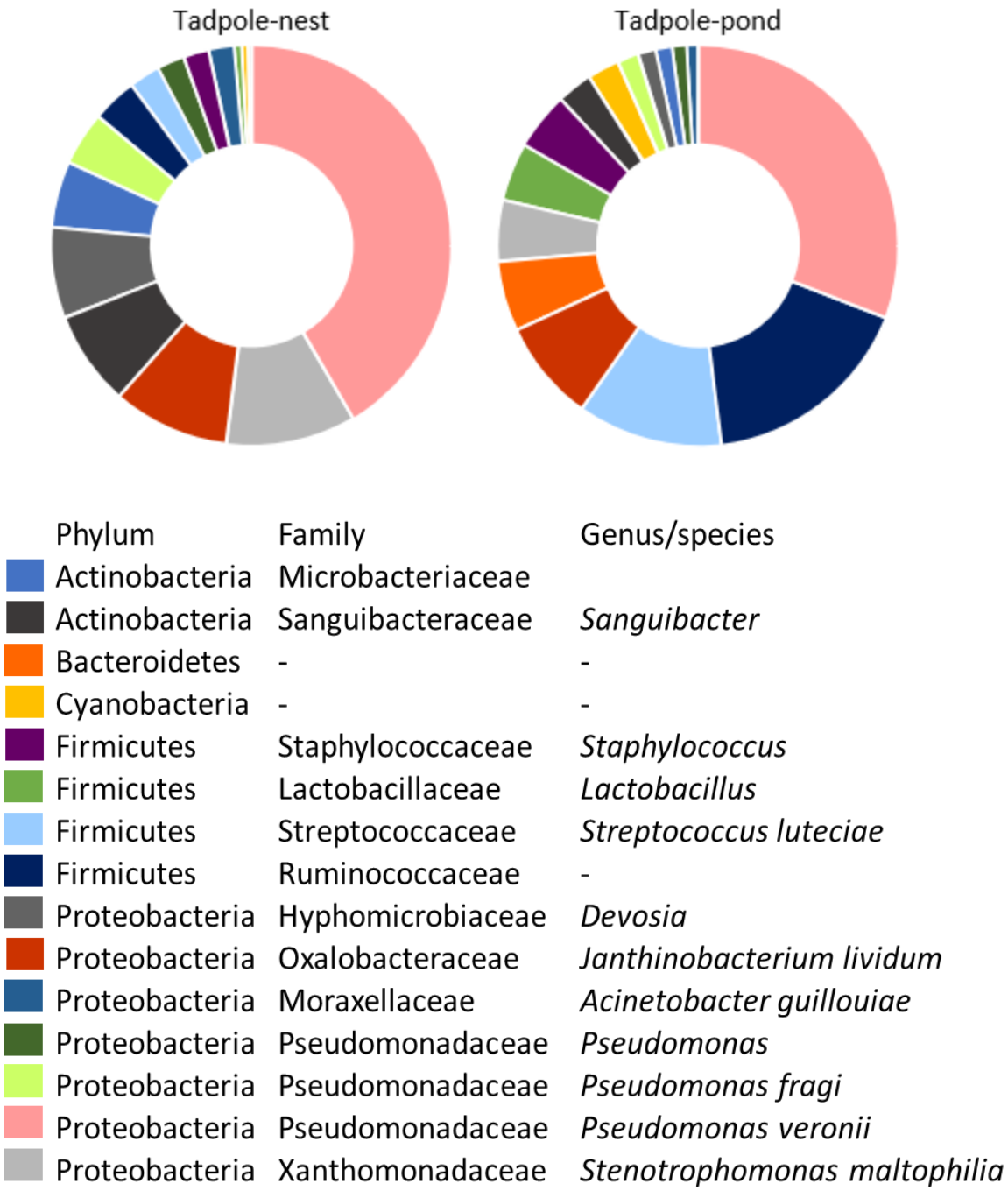


Figure 15. Relative abundance of the tadpole skin core microbiome present 100% of tadpole samples before and after environmental interaction. Legend contains taxonomic designations for all taxa associated with the tadpole core microbiome.

DISCUSSION

The skin-associated microbial communities of three pairs of foam nesting frogs from the genus *Polypedates* exhibit diversity and variability across an initial developmental gradient. Microbial communities were similar in taxonomic presence and relative abundance across all adult individuals, regardless of the species, sex, or body location sampled, suggesting a ubiquitous adult microbiome among members of the genus *Polypedates* in Borneo. Previous studies, however, have shown significant differences in microbial community composition between host species (6, 8, 97) and among body locations sampled (98). Although there appears to be a species-specific difference in our results, we cannot support this statistically because of low sample size (i.e., 3 nests and 6 individual adults in total). We predict that with more intensive sampling significant effects of species and body location will be found. Similar skin microbiomes between male and female adult frogs suggest no sexually dimorphic differences in microbiota acquisition or selection, however, no research has been conducted specifically delineating skin microbiome differences related to sexual dimorphism in amphibians, which would be an intriguing future study, particularly in the context of microbial transmission.

Interestingly, but not surprisingly, the inside and outside of the foam nests exhibit large differences, with greater variability among microbial taxa seen inside the nest, tightly corresponding to nest number/species while remaining distinct from the reliably similar nest outside. These significant differences between foam nest insides perhaps underlie a potential trend of species specific microbiome compositional differences, which further sampling might determine, but are so amplified in these samples that the

inside foam nest microbiome seems to be an important factor in terms of providing a specific microbial habitat for offspring. More research is needed to determine if vertical transfer of protective microbiota is occurring via these nests or if inside nest microbial variability lends itself towards adaptability of nests/tadpoles in different environments, however, these preliminary results show strong coupling and suggest a significant need for further research in this area.

In comparison of vertical and environmental microbial transmission on the tadpole skin microbiome; adults, foam nests, and tadpoles sampled before and after environmental interaction all differed significantly from environmental samples. Adults and tadpoles extracted from the nest showed similar microbial taxonomic composition and relative abundance (Figure 7; Table 1), which provides evidence for a stronger influence of vertical microbial transfer on the tadpole skin microbiome before they exit the nest. However, the skin microbiome of tadpoles extracted from the nest did not mirror the nest inside environment. Being more similar to the adult microbiome than the nest inside microbiome, even in the midst of higher variability of microbial taxa across inside foam nest samples, suggests the involvement of other microbial establishing processes besides simple microbial transfer. Tadpole specific selective processes or ecological microbial interactions between nest inside microbiota and tadpole skin microbiota might be driving these results, however, further testing is required to determine which processes are the most influential.

The tadpole skin microbiome also experienced a significant shift in microbial composition and relative abundance of taxa after one week of environmental interaction. Interestingly, again tadpole skin microbiota did not shift to mirror their environment, the

pond water. A unique aspect of the tadpole skin microbiome samples collected after environmental influence is that these sequence libraries consistently had much higher abundances of broadly characterized taxa, deemed bacteria, than any of the other samples (Figure 7). As the same protocol was followed across samples, this suggests that these tadpoles either acquired these bacteria from another source, that competitive interactions of skin and environmental microbiota drive these high bacterial abundances, or that they select for this broadly defined bacteria from the small portion in the water to grow at a high abundance on their skin (62). It is probable that these sequences deemed bacteria constitute novel, uncharacterized taxa or perhaps are the residual effect of taxonomic ambiguity sometimes associated with 16S sequencing analyses (70). While lumped into the same bacterial category, multiple taxa may actually be present within this broad designation. As this study provided only snapshots of tadpole skin and water environment microbiomes, a more comprehensive temporal study would be required to determine variability of microbiota within these systems, as is naturally found in other environmental systems (99), and more sampling is required to elucidate the influences driving these shifts in microbial diversity and relative abundance.

Core microbiome analyses across all variables and focusing on tadpole skin microbiome comparisons allowed for identification of specific taxa of interest while highlighting commonalities between microbial communities, however, these core thresholds were user defined (i.e., 85% of all samples and 100% of tadpole samples, respectively) and should be considered non-exhaustive, as multiple defining characteristics can be used to determine the "core microbiome" (100). Foam nest core microbiome analysis showed a high prevalence of *Acinetobacter guillouiae*, a known

algicidal and electrochemically active bacterium (101–103), which might play a role in foam nest consistency or function. Little is known about foam nest structure, other than one study looking at protein composition of *Polypedates leucomystax* foam nests, of which the majority were enzymatic or structural proteins with one anti-microbial peptide present (104). Therefore more research into foam nest composition, microbial ecology, and functional capability should be conducted using a multi-omics approach (51) to delineate the role that nest properties and microbiome have on tadpole offspring. The tadpole-associated core microbiome did exhibit a presence of the bacterium, *Janthinobacterium lividum*, an inhibitory bacterium of the chytrid fungus (43). This may not preclude anti-chytrid bacterial function in this amphibian system, as *Janthinobacterium lividum* was found to be ineffective at establishing long term on species other than Plethodontid salamanders without the aid of antibiotics (45) and the bacterium may not persist through the metamorph and adult stages. However, this indicates the strong potential for anti-fungal bacterial presence in this system and future research into anti-fungal metabolite producing species is highly recommended.

Overall, our findings suggest that both vertical and environmental transmission influence the tadpole skin microbiome and therefore both avenues should be considered when pursuing efforts in amphibian conservation. Vertical transmission seems to play an important role in establishing the original microbial community of amphibian offspring, however, the tadpole microbiome does shift after environmental influence, and more research is needed to fully determine the driving forces of this shift. While low sample size and relatively low sequencing depth (e.g., 4,189 lowest reads per sample compared to 12,335 lowest reads per sample (105)) may have contributed error to these trends,

other studies with rarefied reads per sample similar to our lowest read counts (7) and high sequence quality affirm sequence viability. Due to these discrepancies, however, these data should be considered preliminary evidence of microbial transmission in this system and future research in this area should contain more robust sample and sequence counts to further investigate these trends. Also, further research examining temporal variation at a multitude of time-points would assist in visualizing environmentally driven shifts and looking at the amphibian skin microbiome across metamorphosis into adulthood would also help complete the microbiome characterization for these species. In addition, further investigation into the effects environmental change has on the amphibian skin-associated microbiome is needed to more fully delineate the role environment has on the microbiome. This is particularly important for island geographic regions, where climate change and sea level rise pose exacerbated threats (106).

Amphibian skin-associated microbiomes are diverse communities that play an important role in host health (13, 107, 108). Microbial transmission is important to delineate in order to understand how amphibian offspring acquire their initial microbiome, which can have profound health effects for them later in life (12, 44, 107, 108). Gaining an understanding of the processes attributed to microbial acquisition can aide in amphibian conservation efforts, particularly those focused on probiotic efforts to stem the effects of chytridiomycosis, as well as grant us a greater understanding of our natural world. The conservation implications of this study also may be transferrable to other tropical areas, including the New World tropics and Africa, where species convergent for this breeding strategy exist (109, 110). This type of exploration is novel for old-world tropical amphibian species and should be expanded within this region.

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Appendix

Characterization of adult *Polypedates leucomystax* pair associated with unfertilized foam nest and unfertilized nest microbiome. An adult pair was captured by other field scientists as part of another study at the same time and location of sample collection for the study detailed above. The male and female were placed in separate plastic bags and the female produced a foam nest in the bag by herself, not having been in contact with the male during the formation of the nest. These adults, as well as the resulting infertile nest, were sampled and sequenced to serve as a comparison of nests made by pairs versus that made by a single female individual. The infertile foam nest was sampled approximately six hours after formation of the nest by swabbing for 15 seconds, along with both of the adults. Adults were sampled using the same protocol as all other adult pairs and the nest was sampled in triplicate. Sequence processing and data analysis followed the same protocol as the above study.

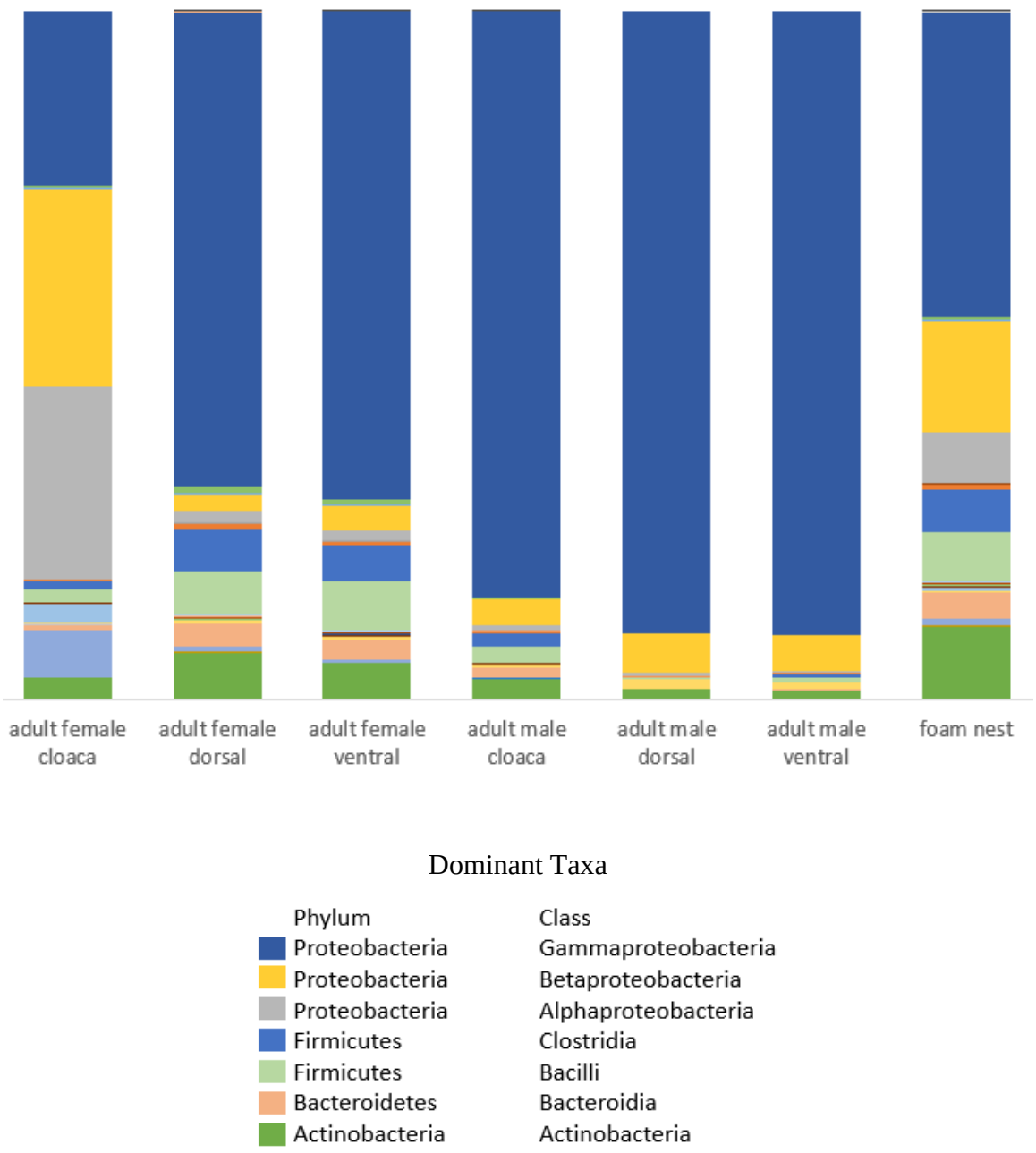

Figure A1. Taxonomic bar plots depicting the bacterial community composition of *Polypedates leucomystax* adults by body location sampled and unfertilized foam nest.

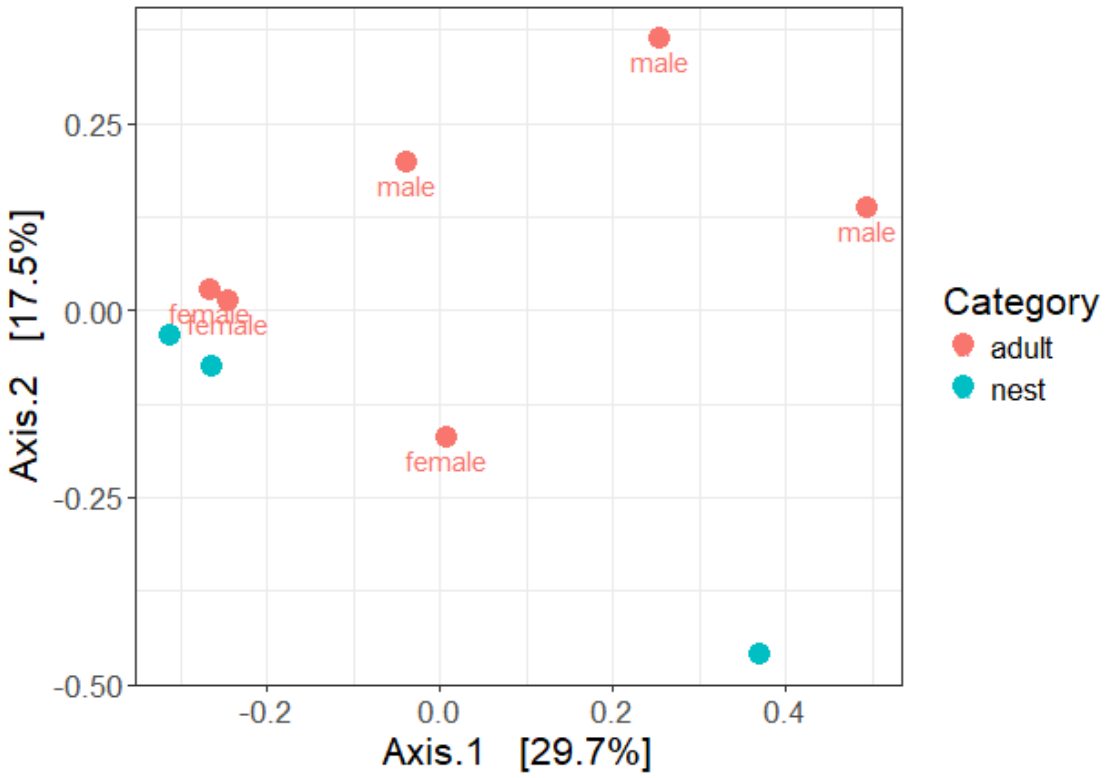

Figure A2. Principle coordinate analysis of adult frogs by sex and unfertilized foam nest using a Jaccard distance matrix (presence/absence of taxa).